REMARKS

Claims 10, 13-18, 31, and 33-40 are under prosecution. Claims 1-9 are withdrawn from consideration. Claims 11-12, 19-30 and 32 are canceled. Claims 10 and 31 have been amended. New claims 38-40 are added. No new matter is added. Applicants respectfully request reconsideration of the rejections.

The Office Action states that the present application lacks the necessary reference to provisional application 60/129,449 as claimed in the Declaration. The application has been amended to recite the appropriate reference. The reference has further been amended in accordance with the filing receipt to reflect the complete lineage of USSN 09/356,322. Applicants agree to provide a substitute Declaration upon indication of allowability of the claims.

Claims 10-16, 18 and 31-37 have been rejected under 35 U.S.C. 102(e) as anticipated by Brown *et al.*, U.S. Patent no. 5,807,522, issued September 15, 1998. The present application claims priority to U.S. patent application no. 08/261,388, filed June 17, 1994, which application discloses the invention of Claims 10-16, 18 and 31-37. The cited U.S. Patent no. 5,807,522 claims priority to the same application. Inventors Tidhar Shalon and Patrick Brown are common to both the cited patent and to the present application. Inventor Patrick Haab provided improvements relating to the invention of Claim 19, which claim is deleted herewith. A request for correction of inventorship accompanies this response. Therefore the '522 patent does not have an earlier effective U.S. filing date, and is not prior art to the present application under 35 U.S.C. 102(e). Withdrawal of the rejection is requested.

Claims 10, 11, 13-15, 18, 31, 33-35 and 37 have been rejected under 35 U.S.C. 102(b) as anticipated by Chang *et al.*, U.S. Patent no. 4,591,570, issued May 27, 1986. Claim 31 has been amended to incorporate the limitations of Claim 32. The cited reference therefore does not disclose all the limitations of the present claim and cannot anticipate the invention as set forth in Claim 31. Pending claims 10, 13-18 and 33-37 depend on Claim 31, and therefore are not anticipated. Withdrawal of the rejection is requested.

Claims 10-15, 18, 31-35 and 37 have been rejected under 35 U.S.C. § 103(a) as obvious over Beattie (U.S. Patent No. 5,843,767, filed April 10, 1996) for the asserted reason that Beattie, although

using a different method to make the microarray, discloses a microarray of polypeptides deposited at defined positions on a solid support, which makes the claimed invention obvious. Applicants respectfully submit that the presently claimed invention is not made obvious by the cited combination of references.

The Office Action states that Beattie teaches a microarray comprising binding reagents deposited at defined positions on a planar solid support, and cites claims 1 and 15 of Beattie in support of this position. Applicants respectfully submit that Beattie fails to teach a microarray comprising binding reagents deposited at defined positions on a planar solid support. As will be demonstrated by a reading of the Beattie claims and specification, the prior art support is not a solid material, it is not planar, and the regions where binding reagents are immobilized are the channel walls, not a planar surface.

The Office Action has cited Claim 1 of Beattie, which must be read in its entirety:

1. A device for binding a target molecule, comprising:

a substrate having oppositely facing first and second major surfaces;

a multiplicity of discrete channels extending through said substrate from said first major surface to said second major surface;

a first binding reagent immobilized on the walls of a first group of said channels, and

a second binding reagent immobilized on the walls of a second group of said channels.

Applicants note that the substrate of Beattie is not a solid planar surface, but a substrate comprising (as recited above in Claim 1) "a multiplicity of discrete channels", and where the binding reagent is not on the flat surface but on the walls of the channels, which are curved surfaces. As stated by Beattie, a "variety of materials can be immobilized or fixed to the glass surfaces within the channels of the NCG array, to yield a high surface area to volume ratio[1]", which is not found in a planar surface.

Further, as described in the specification, the invention of Beattie is a "flow-through" sensor, designed "such that said test sample upon contact with said substrate is capable of penetrating therethrough during the course of said binding reaction". It is therefore integral to the Beattie invention that the substrate comprises, not a solid material, but a porous material, and that the substrate not exist as a planar solid, but as a microfabricated channeled device.

Beattie specifically teaches away from the use of flat, *i.e.* planar, substrates, stating that "Another limitation of these prior art approaches is the fact that a flat surface design introduces a rate-

[1] column 9, lines 57-59)

limiting step in the hybridization reaction, i.e., diffusion of target molecules over relatively long distances before encountering the complementary probes on the surface. In contrast, the microfabricated apparatus according to the present invention is designed to overcome the inherent limitations in current solid phase hybridization materials, eliminating the diffusion-limited step in flat surface hybridizations and increasing the cross sectional density of DNA.[2]"

Clearly, the requirements for producing a microfabricated, channeled array are very different from that of producing a planar array. While the Beattie arrays require a demanding fabrication procedure in order to obtain a substrate, for example the nanochannel glass arrays described at column 9, line 46 to column 10, line 9. The microarrays of the present invention provide an alternative mode of production and end-product, which can be produced on a simple glass slide. The many advantages of the methods of the present invention can be seen in the current wide spread use of Applicants' technology.

Attached is a recent "Perspective on Protein Microarrays"[3]. The article states that "using technologies employed in the manufacture of DNA microarrays, a glass slide (the "chip") could be printed with thousands of protein detectors", and that "most arrays are created on glass or silicon slides." The simplicity and ease of Applicants' method are clearly favored, and have provided for the market success of those using these methods. Indeed, the milestone publication of a microarray of a yeast proteome (Zhu *et al.* (2002) Science 293:2101, attached) uses the methods as set forth by Applicants, in spotting proteins onto a glass slide using a commercial microarrayer.

The Examiner's attention is further drawn to newly added Claims 38-40, which recite a microarray consisting substantially of a planar solid support comprising 1000 or more discrete regions of distinct polypeptides per cm^2, which microarray is clearly not taught or suggested by the channeled arrays of Beattie.

Applicants respectfully submit the presently claimed invention is not taught or suggested by Beattie. Withdrawal of the rejection is requested.

Claims 16 and 36 have been rejected under 35 U.S.C. § 103(a) as being unpatentable over Beattie as defined by Zubay as applied above, and further in view of Van Ness *et al.* The Office Action states that Beattie teaches a microarray comprising binding reagents deposited at defined positions on

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[2] (column 3, lines 18-26)

[3] Mitchell (2002) Nature Biotechnology 20:225

a planar solid support, but do not teach a cationic film on the solid support. Van Ness et al. specifically teach a cationic film for convenient attachment of polypeptides.

Applicants respectfully submit that the secondary reference does not correct the deficiencies of the primary reference. Van Ness et al. fails to teach or suggest a planar microarray comprising at least 10^3 different polypeptides/cm^2, and there is no suggestion that the methods of polypeptide attachment could be used in the methods as claimed by Applicants.

As demonstrated above, neither Beattie nor Van Ness teach or suggest microarrays on solid planar substrates. Applicants respectfully submit the cited combination of references do not make obvious the presently claimed invention. Withdrawal of the rejection is requested.

Applicants submit that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number STAN-128.

Respectfully submitted,
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Global Analysis of Protein Activities Using Proteome Chips

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To facilitate studies of the yeast proteome, we cloned 5800 open reading frames and overexpressed and purified their corresponding proteins. The proteins were printed onto slides at high spatial density to form a yeast proteome microarray and screened for their ability to interact with proteins and phospholipids. We identified many new calmodulin- and phospholipid-interacting proteins; a common potential binding motif was identified for many of the calmodulin-binding proteins. Thus, microarrays of an entire eukaryotic proteome can be prepared and screened for diverse biochemical activities. The microarrays can also be used to screen protein-drug interactions and to detect posttranslational modifications.

A daunting task after a genome has been fully sequenced is to understand the functions, modification, and regulation of every encoded protein (*1*). Currently, much effort is devoted toward studying gene, and hence protein, function and regulation by analyzing mRNA expression profiles, gene disruption phenotypes, two-hybrid interactions, and protein subcellular localization (*2*). Although these studies are useful, much information about protein function can be derived from the analysis of biochemical activities (*3–7*). In principle, the biochemical activities of proteins can be systematically probed by producing proteins in a high-throughput fashion and analyzing the functions of hundreds or thousands of protein samples in parallel using protein microarrays (*5, 6, 8*). Major hurdles in screening an entire proteome array have been the ability to generate the necessary expression clones and also the expression and purification of proteins in a high-throughput fashion.

We have constructed a yeast proteome microarray containing approximately 80% yeast proteins and screened it for a number of biochemical activities. We first built a high-quality collection of 5800 yeast open reading frames (ORFs) (93.5% of the total) cloned into a yeast high-copy expression vector using recombination cloning (*9*). The yeast proteins are fused to glutathione *S*-transferase–polyhistidine (GST-HisX6) at their NH_2-termini and expressed in yeast using the inducible *GAL1* promoter (*5, 9*). The yeast expression strains contain individual plasmids in which the correct yeast ORFs have been shown to be fused in-frame to GST by DNA sequencing. The proteins were expressed in yeast to help ensure that the proteins were modified and folded properly. Using a 96-well format, 1152 samples were purified at once from yeast extracts using glutathione-agarose beads (*10*). We included 0.1% Triton in the lysis buffer and washes to ensure that the purified proteins were free of lipids. The quality and quantity of the purified proteins were monitored using immunoblot analysis of 60 random samples (Fig. 1A). Greater than 80% of the strains produced detectable amounts of fusion proteins of the expected molecular weight.

To prepare the proteome chips, we printed 6566 protein preparations, representing 5800 different yeast proteins, in duplicate onto glass slides using a commercially available microarrayer. Our initial experiments used aldehyde-treated microscope slides (*6*) in

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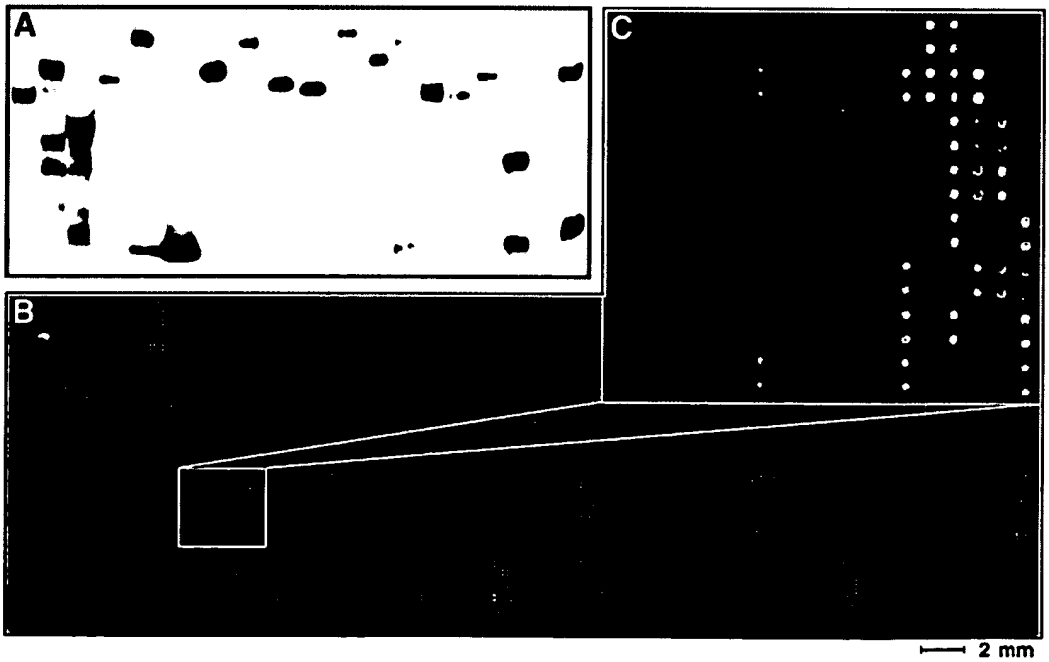


Fig. 1. GST::yeast proteins were purified in a 96-well format. (**A**) Sixty samples were examined by immunoblot analysis using anti-GST; 19 representative examples are shown. Greater than 80% of the preparations produce high yields of fusion protein. (**B**) 6566 protein samples representing 5800 unique proteins were spotted in duplicate on a single nickel-coated microscope slide. The slide was probed with anti-GST (*10*). (**C**) An enlarged image of one of the 48 blocks is depicted to the right of the proteome chip.

which fusion proteins attach to the surface through primary amines at their NH_2-termini or other residues of the protein. In subsequent experiments, we spotted proteins onto nickel-coated slides, in which the fusion proteins attach through their HisX6 tags and presumably uniformly orient away from the surface. Although both slides were successful, the nickel-coated slides gave superior signals for our particular protein preparations (Fig. 1B).

To determine how much fusion protein was covalently attached to different glass surfaces and the reproducibility of the protein attachment, we probed the chips with antibodies to GST (anti-GST). More than 93.5% of the protein samples gave signals significantly above background (i.e., greater than 10 fg of protein), and 90% of the spots contained 10 to 950 fg of protein. Our results also demonstrate that it is feasible to spot, with excellent resolution, 13,000 protein samples in one-half the area of a standard microscope slide (Fig. 1C). To test the reproducibility of the protein spotting, we compared the signals from each pair of duplicated spots with one another; 95% of the signals were within 5% of the average (*10*).

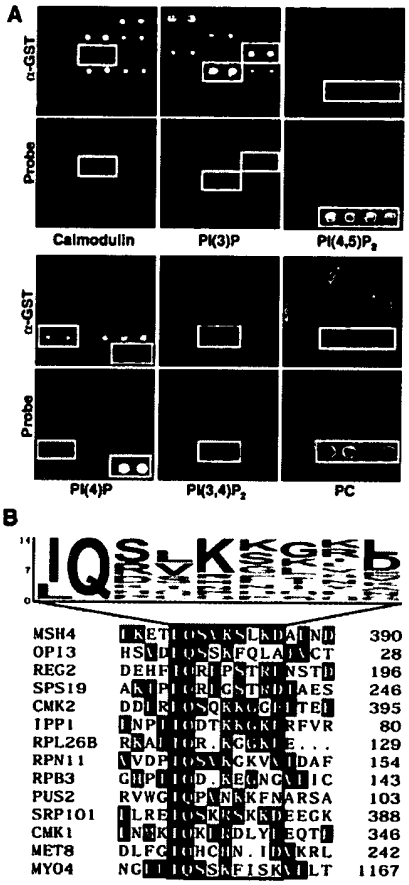


Fig. 2. (**A**) Examples of different assays on the proteome chips. Proteome chips containing 6566 yeast proteins were spotted in duplicate and incubated with the biotinylated probes indicated. The positive signals in duplicate (green) are in the bottom row of each panel; the top row of each panel shows the same yeast protein preparations of a control proteome chip probed with anti-GST (red). The upper panel shows the amounts of GST fusion proteins as detected by the anti-GST (red). (**B**) A putative calmodulin-binding motif (*32*) is shown, which was identified by searching for amino acid sequences that are shared by the different calmodulin targets (*10*). Fourteen of 39 positive proteins share a motif whose consensus is (I/L)QXK(K/X)GB, where X is any residue and B is a basic residue. The size of the letter indicates the relative frequency of the amino acid indicated.

The proteome chips were tested by probing for several protein-protein interactions and protein-lipid interactions. To test for protein-protein interactions, the yeast proteome was probed with biotinylated calmodulin in the presence of calcium (*11*). Calmodulin is a highly conserved calcium-binding protein involved in many calcium-regulated cellular processes and has many known partners (*12*). The bound biotinylated protein was detected using Cy3-labeled streptavidin. As a control, we also probed with Cy3-labeled streptavidin alone. These studies identified six known calmodulin targets (Fig. 2A): Cmk1p and Cmk2p are the type I and II calcium/calmodulin-dependent serine/threonine protein kinases (*12*), Cmp2p is one of the two yeast calcineurins (*13*), Dst1p plays a role in transcription elongation (*14*), Myo4p is a class V myosin heavy chain (*15*), and Arc35p is a component of the Arp2/3 actin-organizing complex (*16*). Arc35p was recently shown to interact with calmodulin in a two-hybrid study (*17*); thus, our data confirm that Arc35p and calmodulin interact in vitro. Of the six known calmodulin targets that we did not detect, two are not in our collection and the rest were not detectable in the GST probing experiments. In addition to known partners, the calmodulin probe identified 33 additional potential partners. These include many different types of proteins [supplementary table 1 (*10*)], consistent with a role for calmodulin in many diverse cellular processes.

Sequence searching (*5*) revealed that 14 of the 39 calmodulin-binding proteins contain a motif whose consensus is (I/L)QXXK(K/X)GB, where X is any residue and B is a basic residue (Fig. 2B). A related sequence in myosins, IQXXXXKXXXR, has been shown previously to bind calmodulin (*18*). Thus, we demonstrate that the domain is found in many calmodulin-binding proteins. Presumably the other targets that lack this motif have other calmodulin-binding sequences (*10*).

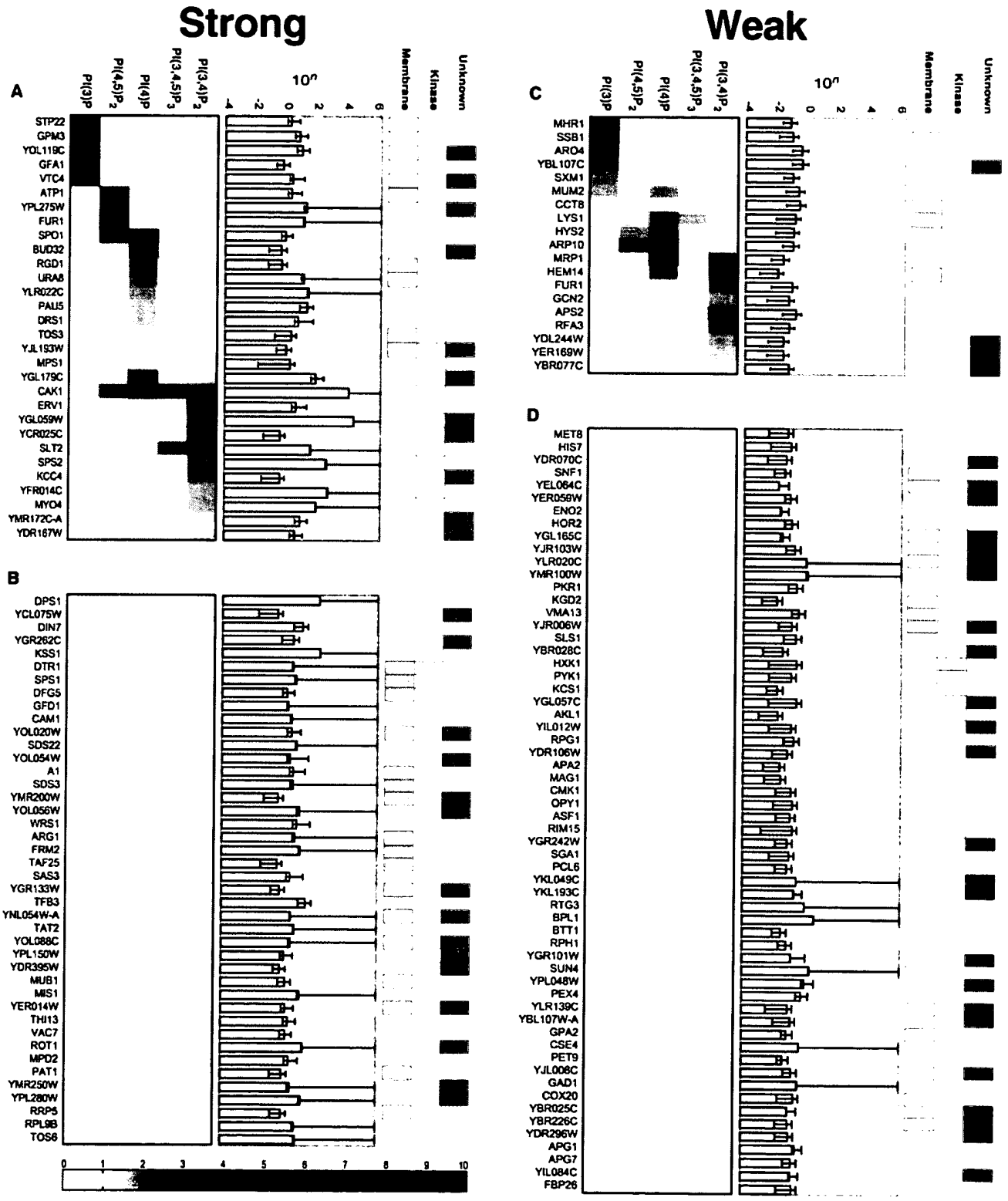
In addition to the calmodulin-binding targets, we also identified one protein, Pyc1p, that bound Cy3-labeled streptavidin. Pyc1p encodes a pyruvate carboxylase 1 homolog that contains a highly conserved biotin attachment region (*19*). Thus, as predicted by its sequence, Pyc1p is biotinylated in vivo. With appropriate detection assays, we expect that proteome chips can identify many types of posttranslational modification of proteins.

To test whether proteome chips could be used to identify activities that might not be accessible by other approaches, such as protein-drug interactions and protein-lipid interactions, we screened for phosphoinositide (PI)–binding proteins. PIs are important constituents of cellular membrane and also serve as second-messengers that regulate diverse cellular processes, including growth, differentiation, cytoskeletal rearrangements, and membrane trafficking (*20*). Because they are often present only transiently and in low abundance within cells, PIs have not been characterized extensively, and little is known about which proteins bind different phospholipids (*20*).

Five types of PI liposomes and one liposome lacking PIs were used to probe the proteome chips. Each contains phosphatidylcholine (PC) with 1% (w/w) *N*-(biotinoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (biotin DHPE); the biotinylated lipid serves as a label that can be detected by Cy3-streptavidin (*21*). In addition to PC, the five other liposomes contain either 5% (w/w) PI(3)P, PI(4)P, PI(3,4)P_2, PI(4,5)P_2, or PI(3,4,5)P_3 (Fig. 2A). All of these phospholipids have been found in yeast except PI(3,4,5)P_3 (*20*).

The six liposomes identified a total of 150 different protein targets that produced signals significantly higher than the background; an algorithm was devised to assist in the identification of positive signals (*22*). Fifty-two (35%) of the lipid-binding proteins correspond to uncharacterized proteins. Of the 98 known proteins, 45 proteins are membrane-associated and either have, or are predicted to have, membrane-spanning regions (*23, 24*).

Fig. 3. Analysis of the PI lipid-binding proteins. To determine the PI-binding specificity of 150 positive proteins, their binding signals were normalized against the corresponding binding signals of PC. On the basis of the ratios (PI/PC), the proteins were grouped into four categories: (**A**) 30 strong and specific, (**B**) 43 strong and nonspecific, (**C**) 19 weak and specific, and (**D**) 58 weak and nonspecific PI-binding proteins. The green color intensity represents the PI/PC signal ratio as shown by the scale in the figure. The first column to the right of the PI/PC binding ratios indicates the maximum binding signal intensity (open boxes) and its confidence interval (solid horizontal lines); the numbers indicate the log of the values. Blue, yellow, light-yellow, and red boxes in columns to the right of confidence interval column identify membrane-associated proteins, protein kinases, other kinases, and uncharacterized ORFs, respectively.


Strong
Weak
A
B
C
D
PI(3)P
PI(4,5)P2
PI(4)P
PI(3,4,5)P3
PI(3,4)P2
Membrane
Kinase
Unknown
STP22
GPM3
YOL119C
GFA1
VTC4
ATP1
YPL275W
FUR1
SPO1
BUD32
RGD1
URA8
YLR022C
PAU5
DRS1
TOS3
YJL193W
MPS1
YGL179C
CAK1
ERV1
YGL059W
YCR025C
SLT2
SPS2
KCC4
YFR014C
MYO4
YMR172C-A
YDR167W
DPS1
YCL075W
DIN7
YGR262C
KSS1
DTR1
SPS1
DFG5
GFD1
CAM1
YOL020W
SDS22
YOL054W
A1
SDS3
YMR200W
YOL056W
WRS1
ARG1
FRM2
TAF25
SAS3
YGR133W
TFB3
YNL054W-A
TAT2
YOL088C
YPL150W
YDR395W
MUB1
MIS1
YER014W
THI13
VAC7
ROT1
MPD2
PAT1
YMR250W
YPL280W
RRP5
RPL9B
TOS6
MHR1
SSB1
ARO4
YBL107C
SXM1
MUM2
CCT8
LYS1
HYS2
ARP10
MRP1
HEM14
FUR1
GCN2
APS2
RFA3
YDL244W
YER169W
YBR077C
MET8
HIS7
YDR070C
SNF1
YEL064C
YER058W
ENO2
HOR2
YGL165C
YJR103W
YLR020C
YMR100W
PKR1
KGD2
VMA13
YJR006W
SLS1
YBR028C
HXK1
PYK1
KCS1
YGL057C
AKL1
YIL012W
RPG1
YDR106W
APA2
MAG1
CMK1
OPY1
ASF1
RIM15
YGR242W
SGA1
PCL6
YKL049C
YKL193C
RTG3
BPL1
BTT1
RPH1
YGR101W
SUN4
YPL048W
PEX4
YLR139C
YBL107W-A
GPA2
CSE4
PET9
YJL008C
GAD1
COX20
YBR025C
YBR226C
YDR296W
APG1
APG7
YIL084C
FBP26

This includes integral membrane proteins, those with lipid modifications [e.g., the glycosylphosphatidylinositol (GPI) anchor proteins Tos6p and Sps2p (*23*) and prenylated proteins (Gpa2p and the mating pheromone a-factor) (*25*)], as well as peripherally associated proteins [e.g., Kcc4p and Myo4p (*15, 26*)]. Eight others are involved in lipid metabolism (e.g., Bpl1p) or inositol ring phosphorylation (e.g., Kcs1p) or are predicted to be involved in membrane or lipid function (e.g., Ylr020cp has homology to triacylglycerol lipase). Of the 52 uncharacterized proteins, 13 (25%) are predicted to be associated with membranes (*24*) and others contain basic stretches, as might be expected for electrostatic interactions with negatively charged lipids. Surprisingly, 19 of the lipid-binding proteins are kinases, and 17 of these are protein kinases.

The phospholipid-binding proteins were sorted into whether they bound lipids strongly or weakly, on the basis of the phospholipid-binding signal relative to the amount of GST (Fig. 3) (*22*). We found that more (72%) of the strong lipid-binding proteins (Fig. 3, A and B) were characterized relative to the weakly binding proteins (54%) (Fig. 3, C and D) and more strong lipid-binding proteins are known or predicted to be membrane-associated, relative to the weaker binding proteins (Fig. 3, "Membrane" column). Interestingly, 13 of 17 of the protein kinases bind very strongly to the PIs. We further grouped the proteins by whether they preferentially bound one or more PIs over PC. One-hundred and one proteins bound to PC as well as or nearly as well as to the PIs (PI/PC < 1.3) (Fig. 3, B and D). However, 49 proteins bound to one or more PIs preferentially (PI/PC > 1.3) (Fig. 3, A and C). Analysis of the strong PI-binding proteins revealed that many of them specifically bound particular PIs. For example, Stp22p, which is required for vacuolar targeting of plasma membrane proteins such as Ste2p and Can1p, preferentially binds PI(3)P (*27*). Nine protein kinases specifically bind PI(4)P and PI(3,4)P_2 strongly and one binds these lipids weakly. Atp1p, a subunit of the F1-ATP synthase of the mitochondrial inner membrane, preferentially binds PI(4,5)P_2 (*28*). Sps2p, which is localized to the prospore membrane (*29*), also interacts specifically with PI(3,4)P_2. Preferential binding of Myo4p to PI(3,4)P_2 may be important for its interaction at the cell cortex and/or its regulation. No strong lipid-binding targets were found that specifically bound PI(3,4,5)P_3, although some proteins bound both this lipid and others (Fig. 3). These results demonstrate that many membrane-associated proteins, including integral membrane proteins and peripherally associated proteins, preferentially bind specific phospholipids in vivo.

Several proteins involved in glucose metabolism were identified as phospholipid-binding proteins. This includes (i) three enzymes involved in sequential glycolytic steps [phosphoglycerate mutase (Gpm3p), enolase (Eno2p), and pyruvate kinase (Cdc19p/Pyk1p)], (ii) hexokinase (Hxk1p), and (iii) two protein kinases (Snf1p and Rim15p). Although unexpected, previous studies indicate that some of these might interact with lipids. Hxk1p binds zwitterrion micelles, which stimulate its activity (*30*), and Eno2p is secreted, suggesting an interaction with membranes (*31*). We speculate that either phospholipids regulate steps involved in glucose metabolism or many steps of glucose metabolism occur on membrane surfaces. In the latter case, the phospholipids would serve as a scaffold to efficiently carry out glycolytic steps.

Six proteins not expected to be involved in membrane function or lipid signaling, Rim15p, Eno2p, Hxk1p, Sps1p, Ygl059wp, and Gcn2p, were further tested for PI-binding using two types of standard assays (*30*). For three proteins, Rim15p, Eno2p, and Hxk1p, PI(4,5)P_2 liposomes were first adhered to a nitrocellulose membrane; different amounts of the GST fusion proteins and a GST control were used to probe the membrane, and bound proteins were detected using anti-GST. As shown in Fig. 4A, each yeast fusion protein tightly bound PI(4,5)P_2, whereas GST alone did not. We also carried out the reverse assay for GST fusion proteins of Rim15p, Sps1p, Ygl059wp, and Gcn2p (*30*). Different amounts of these purified proteins were spotted onto nitrocellulose and probed with the six different liposomes (Fig. 4B); the bound liposomes were detected using a horseradish peroxidase (HRP)–conjugated streptavidin. As with the microarrays, liposomes bound to each protein, but not the bovine serum albumin control. Sps1p bound all five PI-containing liposomes nearly equally. Rim15p, Gcn2p, and Ygl059wp exhibited different affinities to different liposomes (see Fig. 4B for Rim15p); PI(3)P, PI(4)P, and PI(3,4)P_2 bound strongest. In each case, a linear correlation between the binding signal and the level of protein was revealed (*10*). In summary, these results demonstrate that PI-binding proteins identified in the proteome array also bind lipids in conventional assays.

One concern about our experiments is that because proteins are purified from yeast, we might detect indirect interactions through associated proteins. Most of the interactions that we detect are expected to be directly or at least tightly associated with the protein of interest, because proteins were prepared using stringent conditions, and for the seven samples examined, contaminating bands were not detected using Coomassie staining. Another limitation is that properly folded extracellular domains and secreted proteins are likely to be underrepresented in our collection, because GST and a HisX6 tag are fused at the NH_2-terminus. Thus, proteins with a signal peptide may not be delivered to the secretory pathway and may not be folded or modified properly, although we did detect three signal peptide–containing proteins, suggesting that at least some are produced and contain functional domains. Another limitation is that not all interactions are detected because not all proteins are readily overproduced and purified in this high-throughput approach; we expect that 80% of the arrayed yeast proteins are full length and at reasonable levels for screening.

Regardless, the use of proteome chips has significant advantages over existing approaches. Random expression libraries are incomplete, the clones are often not full length, and the libraries are tedious to screen. A recent alternative approach is to generate defined arrays and screen them using a pooling strategy (*4*). The pooling strategy requires two steps, the actual number of proteins screened is not known, and the method does not work well when large numbers of reacting proteins exist, because each pool will test positive. Another method for detecting interactions is the two-hybrid approach (*2*), in which interactions are typically detected in the nucleus, thus limiting the types of interactions that can be detected. The advantage of the proteome chip approach is that a comprehensive set of individual proteins can be directly screened in vitro for a wide variety of activities, including protein-drug interactions, protein-lipid interactions, and enzy-

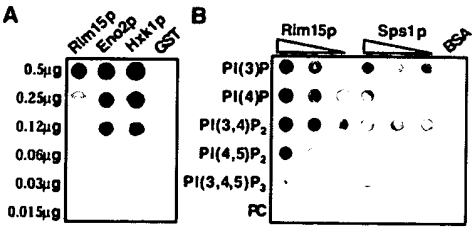


Fig. 4. Conventional methods confirm protein-lipid interactions detected by the proteome microarrays (*30*). (**A**) PI(4,5)P_2 liposomes were first adhered to a nitrocellulose membrane; a dilution series of Rim15p, Eno2p, and Hxk1p, and a GST control were used to probe the membrane. The bound proteins were detected using anti-GST and an ECL kit. (**B**) A reverse assay was carried out to test potential protein-lipid interactions. The proteins were prepared and spotted onto nitrocellulose filters in a dilution series and probed with the six different liposomes. As a control, the six liposomes were also added to the membrane. After extensive washing, the bound liposomes were detected using an HRP-conjugated streptavidin and an ECL kit.

matic assays using a wide range of in vitro conditions. Furthermore, once the proteins are prepared, proteome screening is significantly faster and cheaper. Using similar procedures, it is clearly possible to prepare protein arrays of 10 to 100,000 proteins for global proteome analysis in humans and other eukaryotes.

References and Notes

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10. For details of 96-well format protein purification protocol, a full list of results from all the experiments, and the design of the positive identification algorithms, please visit our public Web site (http://bioinfo.mbb.yale.edu/proteinchip) and supplementary material at *Science* Online (www.sciencemag.org/cgi/content/full/1062191/DC1).
11. Biotinylated calmodulin (CalBiochem, USA) was added to the proteome chip at 0.02 μg/μl in phosphate-buffered saline (PBS) with 0.1 mM calcium and incubated in a humidity chamber for 1 hour at room temperature. Calcium (0.1 mM) was present in buffers in all subsequent steps. The chip was washed three times with PBS at room temperature (RT, 25°C). Cy3-conjugated streptavidin (Jackson IR, USA) (1:5000 dilution) was added to the chip and incubated for 30 min at RT. After extensive washing, the chip was spun dry and scanned using a microarray scanner; the data was subsequently acquired with the GenePix array densitometry software (Axon, USA).
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21. Liposomes were prepared using standard methods (*30*). Briefly, appropriate amounts of each lipid in chloroform were mixed and dried under nitrogen. The lipid mixture was resuspended in TBS buffer by vortexing. The liposomes were created by sonication. To probe the proteome chips, 60 μl of the different liposomes were added onto different chips. The chips were incubated in a humidity chamber for 1 hour at RT. After washing with TBS buffer for three times, Cy3-conjugated streptavidin (1:5000 dilution) was added to the chip and incubated for 30 min at RT.
22. Positives were identified using a combination of the GenePix software which computes a local intensity background for each spot and a series of algorithms we developed. Details can be found at http://bioinfo.mbb.yale.edu/proteinchip and at www.sciencemag.org/cgi/content/full/1062191/DC1.
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An fMRI Investigation of Emotional Engagement in Moral Judgment

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The long-standing rationalist tradition in moral psychology emphasizes the role of reason in moral judgment. A more recent trend places increased emphasis on emotion. Although both reason and emotion are likely to play important roles in moral judgment, relatively little is known about their neural correlates, the nature of their interaction, and the factors that modulate their respective behavioral influences in the context of moral judgment. In two functional magnetic resonance imaging (fMRI) studies using moral dilemmas as probes, we apply the methods of cognitive neuroscience to the study of moral judgment. We argue that moral dilemmas vary systematically in the extent to which they engage emotional processing and that these variations in emotional engagement influence moral judgment. These results may shed light on some puzzling patterns in moral judgment observed by contemporary philosophers.

The present study was inspired by a family of ethical dilemmas familiar to contemporary moral philosophers (*1*). One such dilemma is the trolley dilemma: A runaway trolley is headed for five people who will be killed if it proceeds on its present course. The only way to save them is to hit a switch that will turn the trolley onto an alternate set of tracks where it will kill one person instead of five. Ought you to turn the trolley in order to save five people at the expense of one? Most people say yes. Now consider a similar problem, the footbridge dilemma. As before, a trolley threatens to kill five people. You are standing next to a large stranger on a footbridge that spans the tracks, in between the oncoming trolley and the five people. In this scenario, the only way to save the five people is to push this stranger off the bridge, onto the tracks below. He will die if you do this, but his body will stop the trolley from reaching the others. Ought you to save the five others by pushing this stranger to his death? Most people say no.

Taken together, these two dilemmas create a puzzle for moral philosophers: What makes it morally acceptable to sacrifice one life to save five in the trolley dilemma but not in the footbridge dilemma? Many answers have been proposed. For example, one might suggest, in a Kantian vein, that the difference between these two cases lies in the fact that in the footbridge dilemma one literally uses a fellow human being as a means to some independent end, whereas in the trolley dilemma the unfortunate person just happens to

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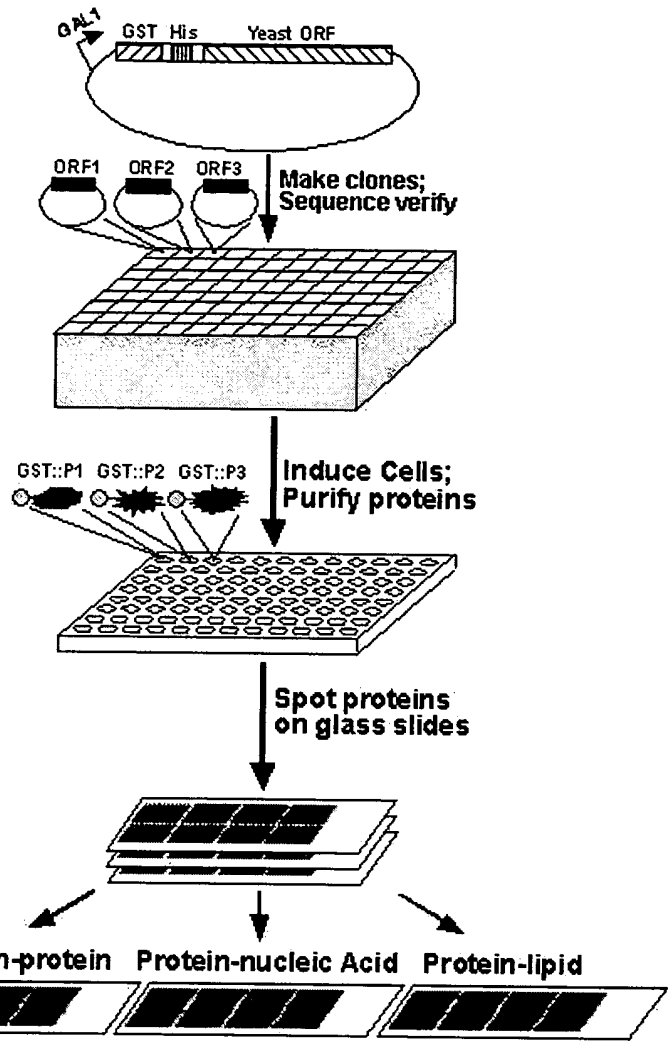

The procedure of yeast proteome analysis using protein chip technology.

The yeast ORFs were cloned into a double tagged yeast GAL1 expression vector via a recombination strategy and verified for correct identities by sequencing. Each pure plasmid construct was then reintroduced into a yeast strain for large-scale protein purification. Yeast cultures were grown in a 96-well format and induced by addition of galactose. After the high-throughput purification step, the purified proteins were aliquoted and stored in a glycerol buffer at ¨C80¡ãC before printing. Using a high precision microarrayer, 6566 protein samples can be double-spotted onto 80 slides in a single experiment.

A perspective on protein microarrays

Proteins, not genes, are the true targets of medicines, but their analysis by array technology still poses significant challenges for drug developers.

Peter Mitchell

Despite great advancements in genomics, researchers remain frustratingly far, in most instances, from implicating specific proteins in disease. Researchers have long known that the concentration of an mRNA within a cell is poorly correlated with the actual abundance of that protein. The only solution is to measure the amount of the specific protein directly. However, when post-translational modifications are taken into account, human cells are a mixing pot of a million or more different protein variants, any of which could be altered in disease. The size of the problem is therefore enormous.

An attractive solution has arrived with the concept of the protein microarray, or protein chip. Using technologies employed in the manufacture of DNA microarrays, a glass slide (the "chip") could be printed with thousands of protein detectors, a biological sample spread over the chip, and any binding noted. The protein of interest could then be analyzed using standard techniques such as time-of-flight mass spectrometry and peptide mass fingerprinting. Such biochips promise a speedy and high-throughput means to profile disease-related proteins or to study protein–protein and protein–drug interactions, previously only achieved using painstaking and tedious standard methods such as western blotting and enzyme-linked immunosorbent assay (ELISA).

With few exceptions, however, the protein chip remains a drug discoverer's fantasy. Despite considerable investment by several companies, protein chips have yet to flood the market. Manufacturers have found that proteins are difficult beasts to handle, and the perceived market demands have not matched those initially anticipated.

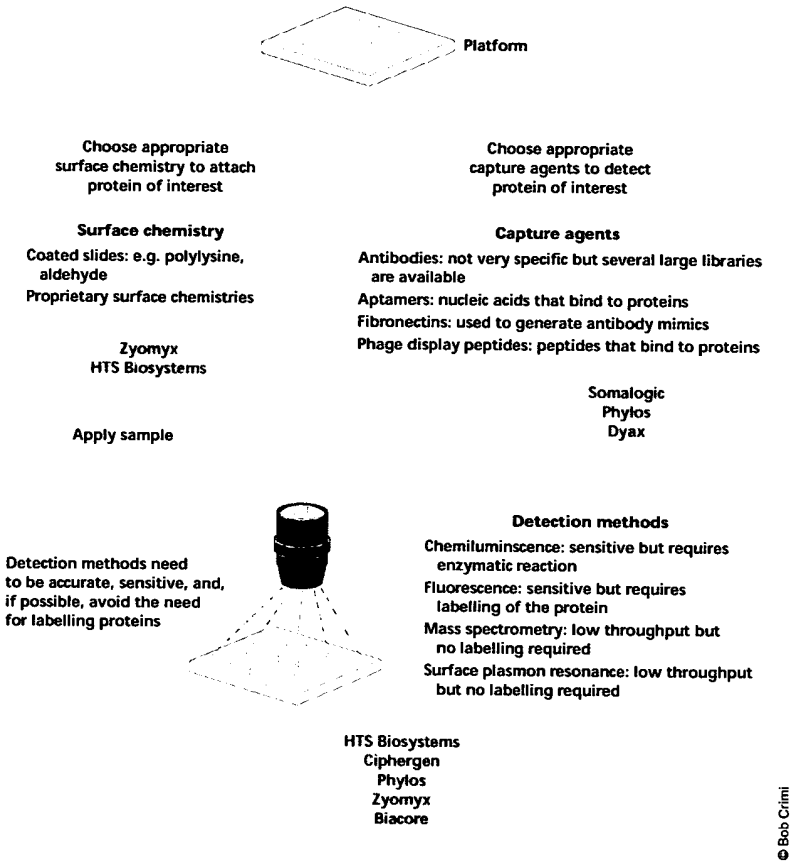

Figure 1. The schematic shows the different stages and components involved in the construction of protein chips. Companies focused on addressing some of these issues are highlighted in the green ovals. Note that many options are still in an experimental stage.

The attraction of arrays

The runaway success of DNA chips in the past five years, with a current market of around $300 million, is undoubtedly responsible for much of the enthusiasm within the protein chip arena.

First, the necessary tools and know-how were already in place: DNA arrayers and contact printers, for instance, could readily be adapted to create arrays of proteins. In theory, protein chips offer customers economies similar to those of DNA microarrays, consuming only tiny quantities of precious and expensive samples and reagents, stocks that might have taken researchers years to prepare. Moreover, from a commercial perspective, protein chips appeared to be a highly lucrative market: In December 2000, market research company BioInsights (Redwood City, CA) predicted that the worldwide market for protein microarrays would grow from $45 million in 2000 to almost $500 million in 2006. Julian Abery, head of Biacore's (Uppsala, Sweden) pharmaceutical and biotech business unit, is even more optimistic, predicting sales of at least $2 billion within the next five years.

However, the analogy with DNA arrays is more apparent than real when it comes to proteins. It has proven far harder to make a marketable protein chip than anyone had expected. Constructing a protein array requires many more steps and more complex science that that used for DNA microarrays.

Among many challenges, to build a viable protein chip a manufacturer needs to:

- choose a surface chemistry that will allow

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immobilized proteins of diverse types to retain their secondary and tertiary structure, and thus their biological activity;
- identify and isolate an agent (e.g., antibody) with which to capture the protein(s) of interest;
- devise a means of measuring the degree of protein binding, assuring both sensitivity and a suitable range of operation;
- in some experiments, extract the detected protein from the chip and analyze it further.

A plethora of different technologies have been promoted, by dozens of companies, to address the needs highlighted in each of these steps (Fig. 1 and Table).

Contact sports

The first challenge is to fix or "print" the protein, or protein ligand, onto the chip in a biologically active form.

Most arrays are created on glass or silicon slides—standard in the production of DNA microarrays—treated with an aldehyde or other agent to immobilize the protein[1].

Other materials being investigated as immobilizing coatings include layers of aluminum or gold, hydrophilic polymers, and polyacrylamide gels. Each material demands its own chemistries (which must avoid denaturing the protein molecules) ideally to orient each molecule in the same direction while at the same time creating a hydrophilic environment in which reactions can take place.

Another alternative is being pursued by Zyomyx (Hayward, CA), which is using photolithography to etch miniature wells on the surface of silicon chips. The immobilized proteins or antibodies are located in the flow chambers on the chip, so that they are always kept in aqueous solution and do not denature through drying-out. The proteins will then be detected by fluorescence labeling, using a rapid-scanning reader developed by the company. Using the photolithography technique, the company can manufacture high-density arrays capable of detecting up to 10,000 proteins in parallel, and will be suitable for target discovery and validation.

For the moment, a rather different approach to chip production has been taken by Large Scale Biology (LSB; Vacaville, CA). Hundreds of thin plastic rods, each doped with a particular antibody, are bundled together in a sheaf. The sheaf is then cut transversely into micrometer-thin slices, yielding chips containing antibody-loaded plastic circles (the rods' cross-sections). A meter-long sheaf could generate a million low-density chips, each with ten to a few hundred elements—potentially a high-volume, low-cost product, says Leigh Anderson, LSB's chief scientific officer (CSO). These chips may never be marketed, however: LSB is developing them for in-house assays and clinical diagnostics only.

Packard (Meriden, CT), also a contender in the protein chip arena, hopes to build on its continuing success in the DNA microarray market. Packard believes its hydrogel chip substrates will be suitable for protein microarrays, and is working with Oxford GlycoSciences (Oxford, United Kingdom) to obtain new capture proteins. Proteins are imprinted on a porous polyacrylamide gel, similar to that used in electrophoresis, and immobilized using a coupling reagent that forms a covalent bond with amine groups on the protein molecules. The gel is aqueous, so immobilized proteins can undergo binding reactions in solution.

Capture agents

Whereas DNA chips use the natural power of hybridization (between complementary nucleotide strands), protein microarrays commonly harness Nature's own protein-capturing agents—antibodies. Slides are printed with an array of different antibodies, which can be monoclonal, polyclonal, antibody fragments, or synthetic polypeptide ligands.

However, huge numbers of antibodies will be needed to populate these chips, and several protein chip companies have been forced to do deals with antibody providers to ensure an adequate supply. For example, Zyomyx has licensed phage-display libraries, which can display hundreds of millions of antibody fragments and proteins, from both Dyax (Cambridge, MA) and Cambridge Antibody Technology (CAT; Cambridge, United Kingdom).

Antibodies are not the only capture agents available: aptamers, oligonucleotides that bind specifically to proteins, can also be used. SomaLogic (Boulder, CO) is constructing arrays using light-sensitive "photoaptamers." The aptamers capture proteins in the sample solution, and the chips are then exposed to ultraviolet light, covalently cross-linking the aptamers with their target proteins. The chips are washed vigorously to remove all unbound protein, and captured proteins are revealed using a universal protein stain.

Aptamer arrays have an advantage over antibody arrays because they can be printed using the same technologies used to create mRNA expression arrays. Manufacturers also claim that they are more specific and sensitive, because the aggressive washing removes much of the unwanted background (aptamer) binding. SomaLogic hopes eventually to put tens of thousands of aptamers onto a chip, although its progress toward this goal was limited by a shortage of proteins to raise aptamer ligands against. For this reason, the company struck a deal with Celera (Norwalk, CT) in which it will give Celera first use of its products in return for proteins and a significant amount of cash backing.

Table: Selected companies working in the protein microarray business

Company (location)	Area of development
Affibody (Stockholm, Sweden)	Antibody-like ligands
Akceli (Cambridge, MA)	Live-cell microarrays
Biacore (Uppsala, Sweden)	Surface plasmon resonance for arrays
Cambridge Antibody Technology (Cambridge, United Kingdom)	Antibodies (phage-display libraries)
Ciphergen Biosystems (Fremont, CA)	Protein-capture chips for mass spectrometry analysis
Dyax (Cambridge, MA)	Antibodies (phage-display library)
HTS Biosystems (Hopkinton, MA)	Surface plasmon resonance arrays
Large Scale Biology (Vacaville, CA)	Plastic-based antibody arrays
Lumicyte (Fremont, CA)	Protein-capture chips for mass spectrometry analysis
Oxford GlycoSciences (Oxford, United Kingdom)	Protein discovery
Packard Bioscience (Meriden, CT)	Gel-based antibody arrays
Phylos (Lexington, MA)	Non-antibody capture agents
Prolinx (Bothell, WA)	Activated-glass array substrates
Protein Sciences (Meriden, CT)	Protein expression
Somalogic (Boulder, CO)	Aptamer arrays
Zyomyx (Hayward, CA)	Silicon-based antibody arrays

Detection is key

The third most pressing need in protein microarrays is detection. How can minute quantities of bound proteins on the chips be detected, bearing in mind that there is no equivalent of PCR for the amplification of proteins? The most obvious method is to use an ELISA-based assay in which the probes are tagged with enzymatic or fluorescent labels. An alternative is to apply a universal protein stain, although this is not an option if the capture molecules are themselves proteins.

There are disadvantages, however, to many of these methods. For example, ELISAs suffer from the nonspecificity of protein–antibody interactions, which cre-

ates "false positives". Labeling proteins with, say, a fluorophore, reduces the quantitative accuracy of assays, because the label can change the way the molecule binds to other molecules. For these reasons, several protein chip companies are using label-free detection systems such as Ciphergen's protein chip, which uses mass spectrometric analysis (see below).

Another unlabeled detection method—surface plasmon resonance (SPR)—is being developed for microarray application by Biacore, HTS Biosystems (Hopkinton, MA), and possibly others.

SPR uses a gold-coated glass chip on which test proteins or antibodies are immobilized. Unlabeled probes—antibodies, proteins from cell lysates, or drug candidates—are applied to the surface, while the chip is scanned from below by a light beam. The beam is reflected back by the gold layer, and the angle of reflection varies according to the mass of the molecules attached. Measuring this angle shows where ligands have bound to the immobilized test proteins. Moreover, it is possible to monitor the kinetics of the reaction in real time, providing a measure of the affinity and speed of binding between the capture agent and probe.

Biacore has marketed SPR-based protein chips for several years, albeit in a single-spot format. In June 2000, it began working with Millennium Pharmaceuticals (Cambridge, MA) to co-develop an array version. "We have prototypes working in labs that measure tens, hundreds, or even thousands of spots at a time," said Biacore's Abery. A commercial product is still at least two years from the market.

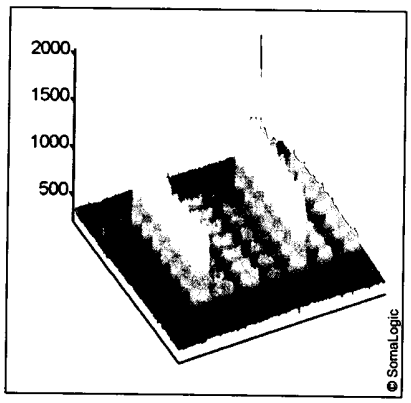
HTS Biosystems has products closer to market, having already shipped several beta versions of its system to Dyax and Mitsubishi Chemical, and a full commercial launch is planned for April 2002. The initial chips will contain 400 protein spots, using antibody assays supplied by Dyax, and future chips could be expanded to thousands of spots, possibly using aptamers and antibodies.

Chips to go

One of the few companies selling commercial protein microarrays is Ciphergen (Fremont, CA). However, its ProteinChips, which are marketed for expression profiling, are unlike most of the others in development. Bill Rich, Ciphergen's chief executive officer (CEO), describes them as "small chromatographic surfaces", carrying either 8 or 16 different spots of capture agents per array. When a biological sample is applied to the chip, the surface chemistry in each spot captures proteins with common properties, such as their affinity for a given ligand.

These spots are then analyzed directly using a laser that evaporates the spot into a benchtop time-of-flight mass spectrometer, a standard protein analysis technique. "By comparing the output from a variant sample and a normal sample, you can see which proteins are differentially expressed," says Rich. The limit of the approach is the resolving power of the mass spectroscopy: "About 1,000 proteins is about the best we can separate in a reasonable amount of time," he says.

Ciphergen has sold over 200 of its systems, generating about a half of the company's 2001 revenues of $19 million—a figure that is set to double in 2002, say analysts. The chips themselves cost $50–$75.



SomaLogic is using its photoaptamers to detect proteins on its chips, providing a simultaneous quantitative measurements of multiple analytes.

In December, Ciphergen introduced a device to interface its chips directly with the tandem mass spectroscopy instruments that are currently used in large protein analysis facilities, allowing for a much more detailed structural analysis of the discovered protein. According to Rich, Human Genome Sciences (Rockville, MD) successfully used this system to examine the structure of the receptor for its protein repifermin, a growth factor now in clinical trials as a wound treatment. "Everybody wants to do this [structure determination] at the moment in drug discovery," he says. "[The chip and detector] is a massive value proposition where only micrograms of the protein are available."

High expectations

Despite these few successes, Steven Bodovitz, a technology analyst at BioInsights, notes how little progress has been made since the company last surveyed the sector in December 2000. "Several companies that announced they were going to launch products in 2001—Somalogic, Phylos, Zyomyx, and HTS Biosystems, among others—but those products did not reach the market."

SomaLogic originally expected to produce 1,000-aptamer arrays by the end of 2001, but has had to delay this. "High-throughput identification of photoaptamers ... took more time than we had hoped," says Larry Gold, the company's CEO. Meanwhile, Zyomyx has not disclosed either the reasons for the delays or what its new timetable might be. Phylos (Lexington, MA) has now dropped plans to commercialize protein microarrays. According to Al Collinson, Phylos' vice president of business development, the decision was made because of the "uncertain dynamics" of the market for protein microarrays. Instead, it will supply its Trinectin ligand technology—based on high-affinity polypeptide "scaffold molecules" such as fibronectin—to a partner who will use the ligands to populate commercial protein arrays.

The bottlenecks ...

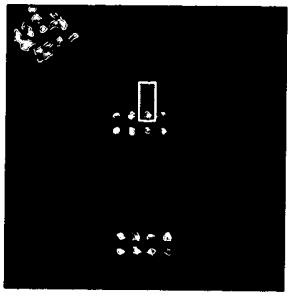
However, perhaps the heart of the problem is that it is fundamentally far harder to work with proteins than with nucleic acids. Bodovitz adds: "Obviously some of the initial enthusiasm was a little ahead of what could actually be delivered." Indeed, there are still many outstanding obstacles for the industry to overcome, although there appears to be some disagreement as to exactly what those are.

Capture agents. According to Snyder, and many others, the biggest problem for the protein chip market is the shortage of sufficiently specific antibodies or other ligands. "All the antibodies at the moment are in the refrigerators of famous professors," says Ciphergen's Bill Rich. "They may have spent 20 years just getting enough volume of five antibodies." Biacore's Abery agrees: "Getting even a 100 proteins in a format pure enough to put on an array is a significant investment."

Moreover, most potential buyers of protein chips do not want off-the-shelf arrays—they want to put their own precious antibodies or target proteins on a chip surface. Array makers must therefore work closely with pharmaceutical partners to build access to useful proteins and ligands.

Currently, off-the-shelf chips have just a few capture agents, typically aimed at a well-defined class of proteins (e.g., cytokines). "For a target-validation assay, you don't need too many proteins on a chip," says Bodovitz. "If you are looking at cancer genes, cytokines, or signal-transduction proteins, you can just study a smaller subset of them at much lower density. The anti-

Microarrays go live



More exotic variants on the protein chip theme include the "living protein chip". David Sabatini, of the Whitehead Institute for Biomedical Research (Cambridge, MA), affiliated with the Massachusetts Institute of Technology (MIT), spotted cDNA onto glass slides and cultured human embryonic kidney (HEK) cells on the surface. The HEK cells take up the cDNA, expressing the corresponding proteins, and can then be used for functional studies of, for example, the interactions of the protein with small molecules (ref. 4, and see *Nat. Biotechnol.* **19**, 535, 2001).

According to Sabatini, living arrays have the advantage that the proteins do not need to be purified, because they are expressed at concentrations so high that they swamp any signal from the cell's endogenous proteins. Also, cells can express any protein, even membrane proteins like G-protein-coupled receptors (GPCRs), which are otherwise difficult to solubilize.

Under a license granted by MIT, the technology will be commercialized by a start-up company called Akceli (Cambridge, MA). Akceli's arrays, which could be launched next year, will be populated with thousands of cDNAs—a significant investment, as full-length cDNA constructs are time-consuming to make and expensive to buy.

However, Sabatini is more interested in using living protein chips for "expression monitoring", examining the effects of expressed proteins on the cells themselves (e.g., to identify proteins that induce apoptosis). Here the arrays, bearing 10,000–15,000 cDNAs on a single slide, provide much faster throughput than a traditional microtiter plate, and allow for direct examination of the cells, for example under a fluorescence microscope. *PM*

bodies for these tend to already exist, and you don't have to worry so much about cross-reactivity."

Because antibodies are generally glycosylated, and have large surface areas for interactions, they show significant cross-reactivity between target proteins. This leads to large numbers of false positives and negatives. So, many companies are trying to develop ligands with better specificity, such as aptamers or Phylos's non-antibody-based peptide scaffolds.

Additionally, many proteins appear in complexes, and it is therefore necessary to find a ligand that can bind to the protein in this new form. "There are several diseases that are related to the composition of a complex variant protein," says Stefan Stahl, CSO of the ligand company Affibody (Uppsala, Sweden). "This must be very difficult to understand with protein chips."

Others, however, such as Kevin Johnson of antibody maker CAT, disagree: "We have got antibodies working on all the platforms we have tried, so there doesn't yet seem to be a technological limitation that says antibodies are not going to be a good idea as content for microarray platforms."

The nature of proteins. Other problems may result in attempts to "stick" proteins on microarray surfaces: proteins often denature at solid–liquid and liquid–air interfaces, and a small protein spot has a disproportionately large surface area, further encouraging denaturation. Furthermore, the surface chemistry *per se* may denature proteins or result in loss of activity. The problem is confounded as the number of different protein spots on the chip increases, because they may not all behave alike when exposed to the same surface chemistry. "You need the proteins in a functional format, and for 100 proteins there may be 100 different ways you need to immobilize them," says Abery.

Optimizing these chemistries demands know-how in many fields, according to Zyomyx, one of the most advanced of the protein chip companies: the solution could include organic thin films, self-assembled monolayers, nanotechnology, biomaterials, and surface biochemistry. Because surface chemistries are key to products reaching the market early, companies are understandably keen to keep trade secrets, and there is little detail available on the exact nature of these products.

Solubility is also a serious difficulty. "Many proteins that are soluble in their native environments might not be soluble when you want to measure them on the chip," says Stahl. Should the chemical environment change, soluble proteins may precipitate onto the chip, wrecking the assay.

Moreover, G-protein-coupled receptors (GPCRs), which make up a large percentage of all targets for current therapeutics, are particularly a problem, because purifying these proteins with detergents can destroy their function. "We need improved technology to get them expressed and assayed in a functional state," says Snyder. "Some people have succeeded in doing this, but not in a high-throughput fashion for purifying hundreds of proteins." (See "Microarrays go live".)

David Sabatini, of the Whitehead Institute for Biomedical Research (Cambridge, MA), raises a further obstacle. "Proteins are notoriously unstable," he warns. Unlike DNA microarrays, which have a shelf life of many months, protein arrays may not be stable in the long term, as the proteins lose their native conformation. "The only point in having an array is to do many experiments quickly. If they are so unstable that you need to print more every couple of days, then that limits their utility." The current literature on the issue is inconclusive. "I expect the answer is that we don't know," says Sabatini. "A lot of the proteins on those big arrays have unknown functions, so you could never actually test whether they are still functional."

But CAT's Johnson adds that antibodies are more robust capture agents, and eventually it will be possible to dry and store them at room temperature.

Robust detection. One more difference between protein and DNA microarrays is that protein concentrations in a single biological sample vary far more than do mRNA levels. So protein-chip detector systems must have very large ranges of operation—up to a factor of 10^{14}, compared with 10^4 for mRNA. For example, an antibody with nanomolar affinity for a specific protein would be swamped by target protein present in micromolar concentration. On the other hand, very rare proteins present at picomolar or femtomolar concentrations would not be detected.

"This means you can choose to detect low-abundance proteins or high-abundance proteins, but to fit them all into one chip might be difficult," says Affibody's Stahl. As a result, some chip companies are considering having separate array product lines—some to detect very rare proteins, and others to detect more abundant types.

But according to CAT's Johnson, this "dynamic range" problem is not insuperable. The solution is to estimate the concentration of the target protein in the sample, and then choose an antibody with an affinity that can match that concentration. If necessary, two or three antibodies to the same target, but with different affinities, can be used in different dots on the chip to cover the whole concentration range.

However, even choosing multiple antibodies may not overcome the problems

inherent in detecting proteins modified after translation, for example by glycosylation or phosphorylation. A ligand designed to bind to the unmodified protein will not necessary bind to the ten or twenty different translationally modified variants. "Often the assay produced from the open reading frame protein just does not work with the protein as it appears in real life," says Ciphergen's Rich. "This means that there is a strong probability of a large number of failed ELISA assays."

There are other, perhaps even more serious, criticisms of the currently popular ELISA method of protein detection. In ELISA, the primary antibody is fixed to the chip and captures the target protein from the sample. This complex is then detected either via a label that was attached to the target protein during expression, or by introducing a labeled secondary antibody that will bind to the complex. It turns out to be very difficult to make this method robust in microarrays, where many different ELISAs have to work on the same chip under the same conditions.

"To get quantitative expression profiling from this, you need a way of uniformly labeling the proteins," says Affibody's Stahl. "But this is a major challenge because they have such diverse properties. You would more likely label one protein to 100% and another to only 10%." As a result, he says, a lot of effort is being put into finding smart non-labeled detection systems, such as mass spectrometry, SPR, and (Affibody's own solution) fluorescence resonant energy transfer (FRET; ref. 2).

Small steps forward

It is a formidable list of problems to solve, but at least some of the companies in the game are confident that they can find solutions.

Indeed, the protein chip field was encourage by the publication of a milestone paper by researchers at Yale University (New Haven, CT) and North Carolina State University (Raleigh, NC) in 2001 (ref. 3). The paper proved that it was feasible to make an array large enough to investigate the function of almost the entire yeast proteome.

Thirteen thousand protein samples were spotted onto half the area of a standard microscope slide. Snyder says: "You can globally see all the proteins that bind a particular other protein or lipid or small molecule, so we think they will be enormously powerful for drug discovery." They will also help to elucidate protein function and map out pathways of protein-protein interactions—work that is currently done by two-hybrid assays. But the microarrays, says Snyder, are between ten to a hundred times as fast as two-hybrid methods, because of the very small amounts of material needed.

The bottom line, Johnson insists, is that all the evidence to date says the technology will work. "The real challenge is how to make it commercially attractive," he adds. However, the million-dollar question is whether the products will reach the market this year, says BioInsights' Bodovitz "Without products, there is not much of a market."

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